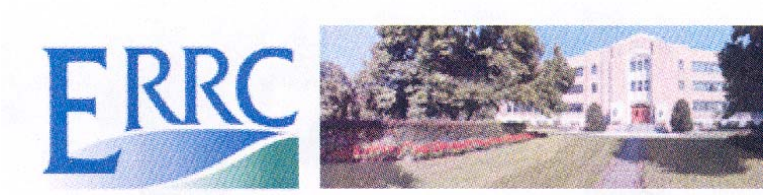

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Composition and Physiological Profiling of Sprout-Associated Microbial Communities

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ABSTRACT

The native microfloras of various types of sprouts (alfalfa, clover, sunflower, mung bean, and broccoli sprouts) were examined to assess the relative effects of sprout type and inoculum factors (i.e., sprout-growing facility, seed lot, and inoculation with sprout-derived inocula) on the microbial community structure of sprouts. Sprouts were sonicated for 7 min or hand shaken with glass beads for 2 min to recover native microfloras from the surface, and the resulting suspensions were diluted and plated. The culturable fraction was characterized by the density (log CFU/g), richness (e.g., number of types of bacteria), and diversity (e.g., microbial richness and evenness) of colonies on tryptic soy agar plates incubated for 48 h at 30°C. The relative similarity between sprout-associated microbial communities was assessed with the use of community-level physiological profiles (CLPPs) based on patterns of utilization of 95 separate carbon sources. Aerobic plate counts of 7.96 ± 0.91 log CFU/g of sprout tissue (fresh weight) were observed, with no statistically significant differences in microbial cell density, richness, or diversity due to sprout type, sprout-growing facility, or seed lot. CLPP analyses revealed that the microbial communities associated with alfalfa and clover sprouts are more similar than those associated with the other sprout types tested. Variability among sprout types was more extensive than any differences between microbial communities associated with alfalfa and clover sprouts from different sprout-growing facilities and seed lots. These results indicate that the subsequent testing of biocontrol agents should focus on similar organisms for alfalfa and clover, but alternative types may be most suitable for the other sprout types tested. The inoculation of alfalfa sprouts with communities derived from various sprout types had a significant, source-independent effect on microbial community structure, indicating that the process of inoculation alters the dynamics of community development regardless of the types of organisms involved.

Alfalfa sprouts are the most commonly consumed green sprouts in the United States. The growing times, temperatures, moisture, and available nutrients with which sprouts are produced favor bacterial proliferation *(18)*. Alfalfa sprouts have been implicated in most of the sprout-associated outbreaks attributable to a variety of *Salmonella* serovars and *Escherichia coli* O157:H7 in the United States from 1995 to 1999 *(9, 18)* and in 2001 (unpublished data). Potential sources of foodborne pathogens include contaminated seeds, irrigation water, poor sanitation, and unhygienic practices at sprouting facilities. Alfalfa seeds are considered the most likely source of contamination for alfalfa sprouts *(17)*. Microbiological analyses have shown that alfalfa seeds may contain high microbial cell densities (10^3 to 10^6 CFU/g *(1, 11, 12)*). Total bacterial counts for alfalfa sprouts increase by 2 to 3 log units during the first day of sprouting and attain a maximum level after 2 days *(3)*. The growth of human foodborne pathogens in these large microbial communities is a major concern because of its potential deleterious effects on human health, especially for very old people, very young people, and immunocompromised individuals.

Physical, chemical, and biological intervention technologies to ensure the microbiological safety of alfalfa sprouts are currently being evaluated. The primary objective of biological intervention technologies research is to develop and optimize competitive exclusion treatments to suppress the growth of human foodborne pathogens on alfalfa sprouts. Ideal candidates for use as antagonists in competitive exclusion treatments are aerobic microorganisms that are easy to culture, nutritionally versatile, and able to proliferate on alfalfa sprouts and outcompete human foodborne pathogens. Organic sprout growers may be able to use biological control interventions to ensure the microbiological safety of their products. The native microflora of alfalfa sprouts may reach cell densities of up to 10^9 CFU/g *(11)*. As part of the overall biological control strategy, the compositions and microbial community structures of native microfloras from various types of sprouts must be assessed. Not only will the use of a biological-control approach involve the manipulation of microbial communities associated with sprouts, but the effectiveness of a potential biological-control agent may be hindered by competition with the native microfloras associated with sprouts.

The main objective of this study was to examine potential sources of variability among microbial communities associated with sprouts to help define the factors affecting the effectiveness of biological-control agents. Sources of variability tested in the present study included (i) sprout type (alfalfa, clover, sunflower, mung bean, or broccoli), (ii) sprout-growing facility (for alfalfa and clover), and (iii)

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seed lot (for alfalfa and clover). Microbial communities on alfalfa sprouts grown from seeds inoculated with microbial communities from various sprout types were compared to evaluate the feasibility of manipulating microbial community composition.

Community-level physiological profiling (CLPP) has been widely used as an easy, rapid tool for assessing the relative similarity between aerobic, heterotrophic microbial communities across spatial, temporal, and experimental gradients *(2, 5, 8, 15)*. The relative similarity of the multivariate profiles of carbon sources generated by whole environmental samples has been shown to be strongly correlated with the relative similarity of community compositions (see summary in Garland *(5)*). While the specific identification of individuals is not possible with it, this approach is particularly useful as a preliminary screening tool to assess the relative effects of various factors on microbial community structure, such as those outlined above (i.e., sprout type, seed lot, growing facility, and inoculation treatment).

MATERIALS AND METHODS

Recovery, enumeration, and identification of bacteria from sprout surfaces. Various types of sprouts were aseptically collected. For the microbial-composition analyses, the sampled sprouts (1.5 g) were submerged in 15 ml of 100 mM potassium phosphate–0.1% peptone buffer (pH 6.8) in a sterile 50-ml centrifuge tube (Corning Inc., Corning, N.Y.) and shaken vigorously by hand for 30 s. The sterile 50-ml centrifuge tube containing the sprout tissues was then placed in a sonicator water bath for 7 min at 20°C. The centrifuge tubes were vortexed for 30 s to disperse the bacteria that were dislodged by sonication. A 0.5-ml sample from the suspension was serially diluted in 0.85% NaCl and plated on tryptic soy agar (TSA; Difco Laboratories, Sparks, Md.) plates. Following incubation at 30°C for 48 h, bacterial colonies were enumerated and phenotypically characterized as described below. For the CLPP analyses, 1.5 g of sprout tissue samples was hand shaken for 2 min in sterile distilled water with glass beads (2 mm in diameter).

Microbial identification of pure isolates derived from sprout-associated microbial communities was carried out with the Biolog Microbial Identification System (Biolog, Hayward, Calif.) as previously described *(6)* and with the Perkin-Elmer MicroSeq 500 16S rDNA bacterial sequencing kit (PE Applied Biosystems, Foster City, Calif.) as previously described *(7, 10)*.

Microbial richness and diversity. On the basis of colony morphology, the number of different types of bacteria (microbial richness) in each sprout surface sample was recorded. The different sprout-associated bacterial colonies grown from sprout surface suspensions on TSA plates as described above were characterized phenotypically to determine the Shannon-Weaver microbial diversity index *(14)*. This index was used as a measure of the level of microbial richness and evenness in sprout-associated microbial communities. Twenty-five bacterial colonies from agar plates containing 30 to 300 bacterial colonies were randomly chosen for the determination of colony morphology. Each colony was given a six-number code based on colony size, pigmentation, form, elevation, margin, and surface. The Shannon-Weaver diversity index (H') is defined as follows: $H' = -\Sigma\ p_i \log_2 p_i$, where p_i is the number of individuals in a cluster (in this case, a species or type of bacteria) divided by the total number of isolates (or types of bacteria) in the sample being analyzed *(14)*.

CLPP. The CLPP assay involves the direct inoculation of environmental samples (e.g., suspensions of bacteria from sprout surfaces) into 96-well Biolog GN2 plates and the subsequent generation of a multivariate profile of sole carbon source utilization based on the reduction of a tetrazolium dye as a result of microbial respiration *(5)*. Sprout suspensions from each sprout type were diluted (1:10) in sterile distilled water to reduce exogenous carbon input to the plates and contaminant reduction of the dye in the control well, which contained no carbon source prior to its inoculation into the microtiter plates (150 μl per well). The plates were incubated in the dark at 25°C for 96 h, and the optical density (590 nm) of each well was measured automatically every 2 h with a Biotek EL320 plate reader (Biotek, Inc., Winooski, Vt.).

Comparison of microbial communities from various sprout types. Alfalfa (The Sproutman), clover (The Sproutman), sunflower (The Sproutman), mung bean (Korean Supermarket, The Sproutman), and broccoli (PC by Formosa Sprouts and/or Fresh Alternatives) sprouts were obtained from ACME Supermarkets (Flourtown, Pa.), Super Fresh Supermarkets (Philadelphia, Pa.), and/or Fresh Fields Supermarkets (Montgomeryville, Pa.). Sprouts were sampled as described above.

Comparison of alfalfa and clover sprouts from different sprout-growing facilities. Alfalfa and clover sprouts were obtained from The Sproutman (sprout-growing facility A; Upper Black Eddy, Pa.), Sunsprout of Lancaster County (sprout-growing facility B; Lancaster, Pa.), East Coast Exotics (sprout-growing facility C; Toughkenamon, Pa.), and Windy Hollow Farm (sprout-growing facility D; Wagontown, Pa.). The sources for alfalfa seeds grown by sprout-growing facilities A, B, C, and D were Ledbetter Farms (lot no. LFF0010), Caudill Seed Co. (lot no. X0141-3), Caudill Seed Co. (lot no. X0141), and Caudill Seed Co. (lot no. KY40203), respectively. The sources for clover seeds grown by sprout-growing facilities A, B, C, and D were International Specialty Supplies, Inc. (lot no. W80CL052), Caudill Seed Co. (lot no. WB-0-CC-054), Caudill Seed Co. (lot no. WB00CC054), and Caudill Seed Co. (lot no. V27001), respectively.

Comparison of sprouts grown from different alfalfa and clover seed lots. Seeds from different sources and from different lot numbers were germinated and grown in the laboratory. Seeds (3 g) were soaked in sterile tap water for 3 h in sterile 8-oz (ca. 237-ml) glass jars. Excess water was removed from these jars and they were placed in an enclosed glass chamber. Sprouts were watered once a day with 3 ml of sterile tap water. Three alfalfa seed sources were tested: International Specialty Supplies, Inc. (lot no. NS9130) (A1), Ledbetter Farms (lot no. LFF0010) (A2), and Caudill Seed Co. (lot no. X0141-3) (A3). Three clover seed sources were tested: International Specialty Supplies, Inc. (lot no. W6-8CC-835) (C1), International Specialty Supplies, Inc. (lot no. W8-0CC-052) (C2), and Caudill Seed Co. (lot no. WB-0-CC-054) (C3). Alfalfa and clover sprouts were harvested on day 7 and the sonication protocol was performed as described above.

Manipulation of microbial community composition. Alfalfa seeds (ORF Certified Organic, lot no. LFF0010) were soaked for 2 h in 30 ml of various suspensions consisting of microbial communities obtained by sonication as previously described. The control treatment consisted of alfalfa seeds soaked in sterile tap water; no sprout-derived microbial suspensions were added. Inoculated alfalfa seeds (3 g) were grown in sterile 8-oz (ca. 237-ml) glass jars. The jars were covered with sterile cheesecloth to allow adequate aeration. The glass jars were placed in an enclosed glass chamber. Sprouts were watered daily with 3 ml of sterile tap

water. Alfalfa and clover sprouts were harvested on day 7 and the sonication protocol was performed as described above.

Statistical analysis. All experiments were performed with a completely randomized design and replicated three times. Depending on the experiment, a replicate consisted of 1.5 g of tissue sampled from the sprouts of a local retailer or a sprout-growing facility or from laboratory-grown sprouts cultivated in 8-oz (ca. 237-ml) glass jars containing 3 g of seeds. Bacterial counts were logarithmically transformed ($\log_{10}$) prior to statistical analysis. Nested analyses of variance were carried out to determine differences between replicate samples and between individual experiments before the results from replicate experiments were combined *(16)*. Significant differences between treatments were determined for a significance level of 0.05 with single and two-way analyses of variance in SAS *(13)*.

The pattern of color development from the CLPP was assessed at an equivalent average well color development to eliminate potential density-dependent bias *(4)*. The individual well responses for an average well color development plate reading of 0.50 absorbance units, corresponding to 40 to 50 h of incubation depending on the sample, were used for subsequent multivariate analysis. Principal components analysis with a covariance matrix was employed to evaluate the relative degree of similarity between samples in multidimensional (i.e., 95 carbon source variables) space (SPSS Software, Inc., Chicago, Ill.).

RESULTS AND DISCUSSION

Identification of bacteria from sprout surfaces. Two microbial identification methods, the Biolog and the MicroSeq methods, were used. We found that the culturable portion of sprout-associated microbial communities included a large number of gram-negative rods. Microorganisms identified included *Pseudomonas fulva, Pantoea ananatis, Pseudomonas veronii, Pseudomonas marginalis, Pantoea dispersa, Pantoea agglomerans, Escherichia hermanii, Pseudomonas synxantha, Pseudomonas syringae tabaci* (pv.), *Pseudomonas fluorescens* biotype A, *P. fluorescens* biotype G, *Pseudomonas asplenii, Erwinia rhapontici, Stenotrophomonas maltophila, Acinetobacter lwoffii, Acinetobacter johnsonii, Enterobacter pyrinus,* and *Pseudomonas putida* biotype A. However, this study represents more a general survey of the type of microorganisms found on sprouts than a descriptive comparison of differences among sprout types, seed lots, or sprout-growing facilities. The level of effort necessary for such a thorough cataloging of organisms from all of the treatments involved was beyond the scope of this work and was the basis for our decision to use CLPP to address our experimental objectives.

Comparison of microbial communities from various sprout types. No statistically significant differences ($P \geq 0.05$) in microbial cell density, microbial richness, or microbial diversity were observed among microbial communities from various sprout types obtained from local retailers. The average aerobic microbial cell density on the sprout surfaces ranged from 7.15 to 8.15 log CFU/g of sprout tissue. Previous studies have reported aerobic plate counts as high as 10^{11} CFU/g *(9, 11, 12, 18, 19)*. The average numbers of morphological types of bacterial colonies observed on the TSA plates ranged from 3 to 5, while the average microbial diversity indices calculated for the surface microbial communities ranged from 2.89 to 3.42.

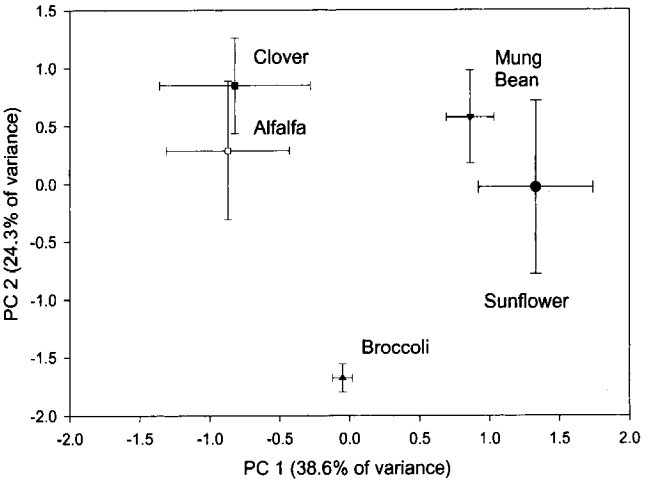

FIGURE 1. *Principal components analysis of CLPP data comparing microbial communities from various sprout types obtained from local retailers. Each point represents the average for three replicate sprout samples; error bars represent ±1 standard error. The percentage of variance explained by principal components analysis is shown on each axis.*

Principal components analysis of the CLPP profiles from the comparison studies among sprout types indicated that the microbial communities associated with alfalfa and clover sprouts are more similar (i.e., closer in principal component space) than are microbial communities from the other sprout types tested (Fig. 1). Microbial communities from mung bean, sunflower, and broccoli sprouts were distinct from each other as well as from alfalfa- and clover-associated microbial communities. The trends observed in patterns of color response are not density dependent, since no statistically significant differences in microbial cell density were found. Instead, these results suggest that the types of bacteria associated with mung bean sprouts (and sunflower and broccoli sprouts to a lesser extent) are distinctive but that the overall numbers of individuals (density) and types (richness) are relatively similar.

Comparison of microbial communities from alfalfa and clover sprouts obtained from different sprout-growing facilities. No statistically significant differences ($P \geq 0.05$) in microbial cell density, microbial richness, or microbial diversity were observed among alfalfa and clover microbial communities on sprouts obtained from four different growing facilities. The average microbial cell densities on the surfaces of alfalfa and clover sprouts from sprout-growing facilities A, B, C, and D ranged from 6.53 to 7.22 log CFU/g of sprout tissue and from 7.05 to 7.52 log CFU/g of sprout tissue, respectively. The average numbers of types of bacteria found to be associated with the surfaces of alfalfa and clover sprouts from the four sampled sprout-growing facilities were 2 and 3, respectively. The average microbial diversity indices calculated for the surface microbial communities on alfalfa and clover sprouts from these sprout-growing facilities were 2.33 and 2.70, respectively.

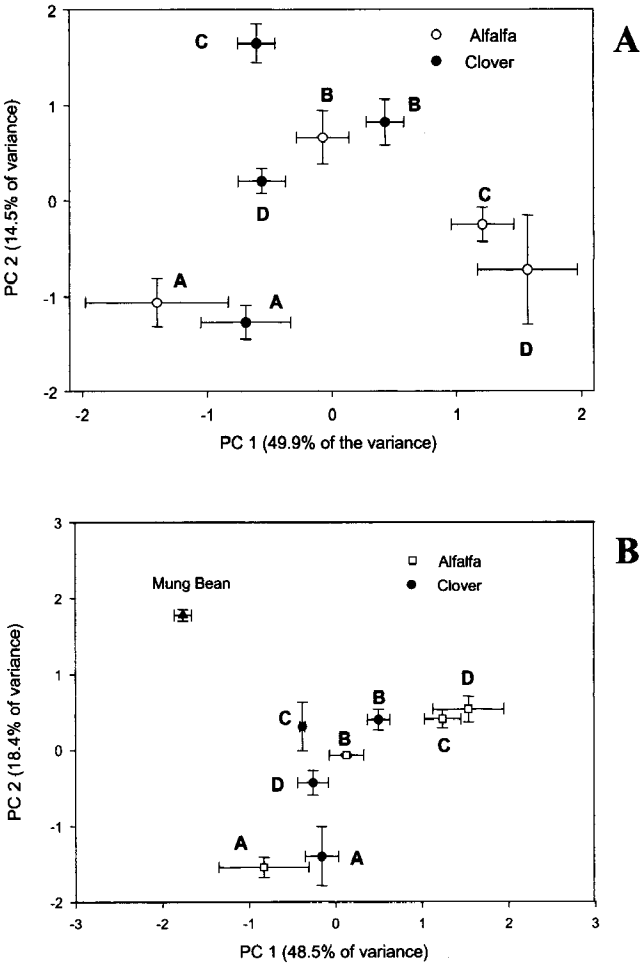

FIGURE 2. *Principal components analysis of CLPP data comparing microbial communities from alfalfa and clover sprouts from different growing facilities without the mung bean sprout reference data point (A) and with the mung bean sprout reference data point (B). Datum points: A, The Sproutman; B, Sunsprout of Lancaster; C, East Coast Exotics; D, Windy Hollow Farm. Each point represents the average for three replicate sprout samples; error bars represent ±1 standard error. The percentage of variance explained by principal components analysis is shown on each axis.*

There were no consistent differences between microbial communities from alfalfa and clover sprouts from different sprout-growing facilities with regard to CLPP (note that A and B samples group close together, while C and D samples do not) (Fig. 2A). Also, there was more extensive site variation among alfalfa microbial communities than among microbial communities from clover sprouts (Fig. 2A). CLPP analyses revealed that the differences among microbial communities from different sprout types were more extensive than differences among microbial communities from alfalfa and clover sprouts from different sprout-growing facilities (Fig. 2B). Note that the CLPP datum point for microbial communities from mung bean sprouts was added as a reference point in the principal components analysis (Fig. 2B). These data support earlier findings that the numbers and types of bacteria associated with alfalfa and clover sprouts are very similar and are distinctive from other sprout-associated microbial communities.

Sources of variation among the different sprout-growing facilities sampled included microbial inocula associated with the facility, the source of irrigation water, the seed treatment prior to germination, the method of sprout germination (rotating drums versus various types of commercial growing trays), the photoperiod, and handling of the sprouts during harvest. All of these factors may be important, but our experimental approach does not allow for specific assessment of them. Our data support the idea that individually or combined, these factors are not as influential (do not cause differences that are as extensive) as the effects of sprout type.

Comparison of microbial communities on sprouts grown from different alfalfa and clover seed lots. Three different alfalfa and clover seed lots were germinated. No statistically significant differences ($P \geq 0.05$) in microbial cell density, microbial richness, or microbial diversity were observed among microbial communities from alfalfa and clover sprouts grown from different seed lots. The overall average microbial cell densities on the surfaces of alfalfa and clover sprouts grown from the seeds of different sources were 8.79 ± 0.07 and 8.75 ± 0.18 log CFU/g of sprout tissue, respectively. An average of 4 ± 1 types of bacteria were found to be associated with the surfaces of both alfalfa and clover sprouts grown from the three seed lots tested. The average microbial diversity index calculated for the surface microbial communities on alfalfa and clover sprouts from different seed lots were 2.80 and 3.00, respectively.

The principal components analysis without the mung bean sprout microbial community reference datum point shows that microbial communities on the surfaces of clover sprouts grown from three different seed lots are similar, while microbial communities on the surfaces of alfalfa sprouts grown from different seed lots are different (Fig. 3A). Microbial communities recovered from the surfaces of alfalfa and clover sprouts grown from three different seed lots are relatively similar to each other (Fig. 3A), but when the mung bean sprout microbial community data are included in the principal components analysis it is clear that the effect of seed lot is less extensive than that of sprout type (Fig. 3B). These data reiterate our findings that the numbers and types of bacteria associated with alfalfa and clover sprouts are very similar and are distinctive from other sprout-associated microbial communities.

Manipulation of microbial community composition. No statistically significant differences ($P \geq 0.05$) in microbial cell density, microbial richness, or microbial diversity were found among alfalfa microbial communities derived from mung bean, alfalfa, clover, and radish sprouts. The average aerobic microbial cell densities on the surfaces of alfalfa sprouts grown from seeds that were inoculated with microbial communities from alfalfa, clover, mung bean, and radish sprouts ranged from 8.90 to 9.54 log CFU/g of sprout tissue, while the average numbers of types of bacteria ranged from 10 to 23. The microbial diversity index values for alfalfa microbial communities ranged from 3.66 to 5.45.

The color response patterns from radish-, alfalfa-, and clover-derived alfalfa microbial communities were very

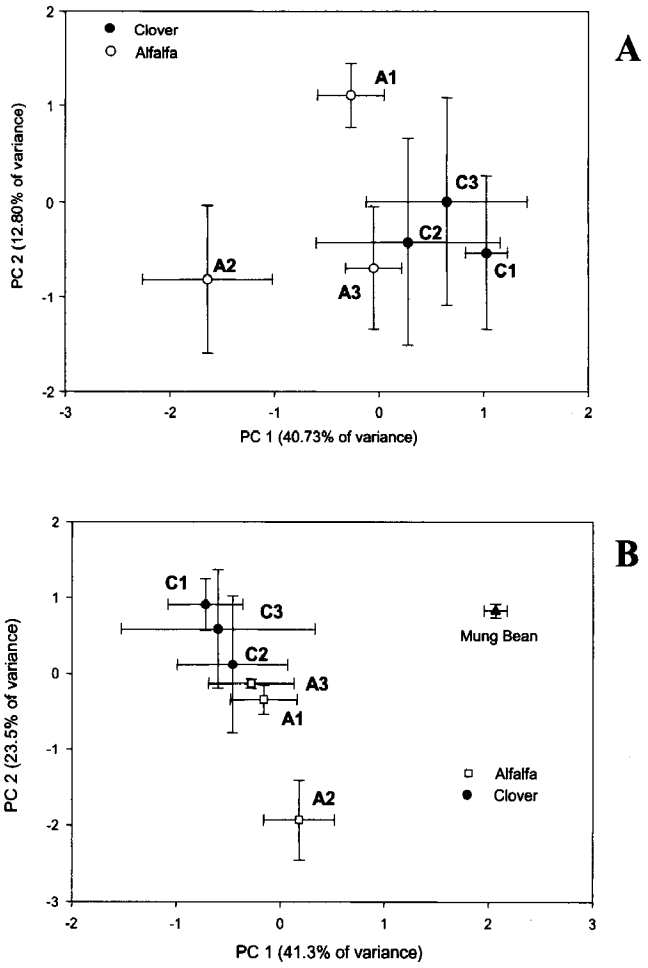

FIGURE 3. *Principal components analysis of CLPP data comparing microbial communities on sprouts grown from different alfalfa and clover seed lots without the mung bean sprout reference data point (A) and with the mung bean sprout reference data point (B). Alfalfa seed sources: A1, International Specialty Supplies, Inc., lot no. NS9130; A2, Ledbetter Farms, lot no. LFF0010; A3, Caudill Seed Co., lot no. X0141-3. Clover seed sources: C1, International Specialty Supplies, Inc., lot no. W6-8CC-835; C2, International Specialty Supplies, Inc., lot no. W8-0CC-052; C3, Caudill Seed Co., lot no. WB-0-CC-054. Each point represents the average for three replicate sprout samples; error bars represent ±1 standard error. The percentage of variance explained by principal components analysis is shown on each axis.*

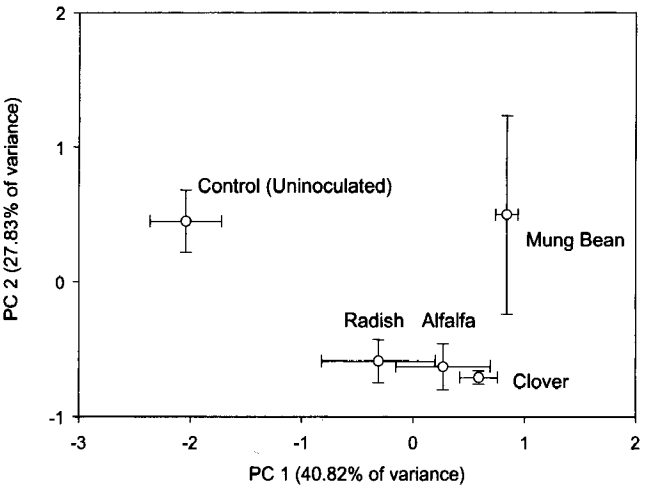

FIGURE 4. *Principal components analysis of CLPP data involving the manipulation of alfalfa sprout microbial community composition. Each point represents the average for three replicate sprout samples; error bars represent ±1 standard error. Each experimental treatment is identified for each point. The percentage of variance explained by principal components analysis is shown on each axis.*

similar, with considerable overlap (Fig. 4). Microbial communities originally derived from mung bean sprouts and inoculated into alfalfa seeds are very distinct (i.e., farther apart in PC space) from microbial communities derived from radish, alfalfa, and clover sprouts. Microbial communities from the uninoculated control are very distinctive from any of the other microbial communities inoculated on alfalfa seeds, suggesting a strong inoculation effect (Fig. 4). Inoculation had a strong effect on the microbial communities sampled even when the inoculum was derived from microbial communities of fully grown alfalfa sprouts, suggesting perhaps that the inoculation effect was not due to the type of microorganisms present but rather to changes in the process of community development.

The results of these studies shed new light on the importance of an assessment of the native microflora as part of the evaluation of potential biological control strategies. The data obtained here indicate that the microbial communities associated with alfalfa and clover sprouts are more similar than are those associated with sprouts of other types, suggesting that perhaps the same biological control agent might be effectively used for these two sprout types. The relative similarity among the microbial communities that develop on alfalfa and clover sprouts grown from different seed lots and by different growers further indicates that the same biocontrol agent may be effective for a broad range of alfalfa and clover sprout growing facilities. Conversely, the distinct CLPPs observed for other types of sprouts (i.e., sunflower, broccoli, and mung bean sprouts) suggest that different biocontrol agents may be better adapted to these sprout types. Finally, the strong effect of inoculation on alfalfa sprout communities, regardless of the source of the inoculum, indicates that the dynamics of community development can be significantly manipulated. Experiments are currently under way to examine various inoculation effects (i.e., type, diversity, density, and time) on the temporal development of sprout-associated microbial communities to help provide information on how altered community development may increase the efficacy of biocontrol strategies.

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